UNIVERSITI PUTRA MALAYSIA

ENZYMATIC ENRICHMENT OF DOCOSAHEXAENOIC ACID (DHA) IN OIL FROM EEL (MONOPTERUS ALBUS)

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By
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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Abstract of the thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

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Chairman : **Professor Mahiran Basri, Ph.D.**

Faculty : **Science and Environmental Studies**

Docosahexaenoic acid, DHA is a polyunsaturated fatty acid that has shown some medical and health benefits At present, the most common sources of DHA are fish lipids The lipids of coldwater fishes are typically high in DHA and these lipids are widely used as food supplements However, for certain pharmaceutical and nutritional purposes, higher concentrations of DHA are required Thus, there is a need to enrich this fatty acid in those lipids which can be done by removal of other fatty acids Enzymatic method is one of the popular methods for DHA enrichment of fish lipids

In this research a local freshwater fish, the eel or *Monopterus albus* was analyzed for its lipid and fatty acid content The fatty acid content of the lipid was analyzed by gas chromatography after it was converted into methyl esters The lipid content of this fish was about 1% and this lipid contained

about 6% of DHA. Subsequently, free fatty acids (FFA) were obtained from this lipid through saponification. The DHA content of this FFA (which was named eel-FFA) was enriched by selective enzymatic esterification with lauryl alcohol. The extent of enrichment of DHA was expressed as "DHA ratio" which is defined as the amount of DHA relative to the total amount of all identified fatty acids in the FFA. The DHA ratio in the original eel-FFA was 0.103. Two immobilized lipases, Novozyme and Lipozyme were tested for their selectivity. These enzymes showed low activity on DHA and thus, DHA was enriched in the unesterified fatty acids fraction. It was found that Lipozyme was more selective than Novozyme and therefore Lipozyme was used in subsequent studies.

Optimization of substrates molar ratio was carried out using FFA : lauryl alcohol molar ratios between 1:1 and 1:6. When the amount of lauryl alcohol was raised from a ratio of 1:1 to 1:2 the increase of DHA ratio in the product was significant. A further increase in the amount of lauryl alcohol didn't give much effect on the DHA ratio. Thus, the optimum molar ratio of FFA : lauryl alcohol was 1:2.

Reactions were also carried out at various temperatures between 30 ^{o}C and 45 ^{o}C. The study showed that reaction temperature did not significantly affect the DHA ratio in the product. Subsequent studies were carried out at 30 ^{o}C.

In the time course study it was found that the DHA ratio increased with time to a maximum of 0.750 after 40 minutes before gradually decreased. When the reaction was carried out for 40 minutes using different amounts of Lipozyme, results showed that a maximum DHA ratio of 0.763 was achieved when 0.4 g Lipozyme for each 0.54 mmol FFA was used. Higher amounts of Lipozyme slightly decreased the DHA ratio.

The DHA-enriched eel-FFA obtained after the reaction at the optimum conditions contained 52% DHA. This was seven times higher than the DHA content in the original eel-FFA which was about 7% DHA.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia bagi memenuhi keperluan ijazah Master Sains.

PENGKAYAAN BERENZIM ASID DOKOSAHEKSAENOIK (DHA) DALAM MINYAK DARIPADA BELUT (*MONOPTERUS ALBUS*)

Oleh

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Asid dokosaheksaenoik, DHA adalah sejenis asid lemak politaktepu yang mempunyai manfaat dari segi perubatan dan kesihatan. Pada masa ini sumber utama DHA ialah minyak ikan. Minyak daripada ikan-ikan di kawasan beriklim sejuk adalah kaya dengan DHA dan minyak-minyak ini digunakan secara meluas sebagai makanan tambahan. Walaubagaimanapun, bagi tujuan farmaseutikal dan pemakanan tertentu, kepekatan DHA yang lebih tinggi adalah diperlukan. Oleh itu, terdapat keperluan untuk memperkayakan asid lemak ini di dalam minyak-minyak tersebut yang dapat dilakukan dengan menyingkirkankan asid-asid lemak yang lain. Kaedah berenzim adalah salah satu kaedah yang popular untuk pengkayaan DHA di dalam minyak ikan.

Di dalam penyelidikan ini sejenis ikan air tawar tempatan iaitu belut atau *Monopterus albus* telah dianalisis kandungan minyak dan asid lemaknya. Kandungan asid lemak di dalam minyak ini telah dianalisis dengan menggunakan kromatografi gas setelah ia ditukarkan kepada ester metil. Kandungan minyak di dalam ikan ini adalah lebih-kurang 1% dan minyak ini mengandungi lebih-kurang 6% DHA. Seterusnya asid lemak bebas (FFA) telah diperolehi daripada minyak ini melalui penyabunan. Kandungan DHA di dalam FFA ini (yang telah dinamakan FFA-belut) telah diperkayakan melalui pengesteran berenzim memilih dengan alkohol lauril. Tahap pengkayaan DHA telah dinyatakan sebagai "nisbah DHA" yang ditakrifkan sebagai amaun DHA berbanding dengan jumlah amaun semua asid-asid lemak yang dikenalpasti di dalam FFA tersebut. Nisbah DHA di dalam FFA-belut yang asal ialah 0.103. Dua jenis enzim tersekatgerak, Novozyme dan Lipozyme telah diuji untuk mengetahui kepilihan masing-masing. Enzim-enzim ini menunjukkan aktiviti yang rendah terhadap DHA dan dengan itu DHA telah diperkayakan di dalam bahagian asid lemak yang tidak mengalami pengesteran. Adalah didapati bahawa Lipozyme lebih memilih daripada Novozyme dan oleh itu Lipozyme telah digunakan di dalam kajian-kajian seterusnya.

Pengoptimuman nisbah mol subsrat telah dilakukan dengan menggunakan nisbah mol FFA : alkohol lauril di antara 1:1 dan 1:6. Apabila amaun alkohol lauril dinaikkan daripada nisbah 1:1 kepada 1:2 kenaikan nisbah DHA di dalam hasil adalah jelas. Pertambahan amaun alkohol lauril

seterusnya tidak memberikan banyak kesan ke atas nisbah DHA. Oleh itu, nisbah mol FFA : alkohol lauril yang optimum ialah 1:2.

Tindakbalas-tindakbalas juga telah dijalankan pada suhu-suhu berlainan di antara 30 ^{o}C dan 45 ^{o}C. Kajian ini menunjukkan bahawa suhu tindakbalas tidak memberikan kesan yang jelas ke atas nisbah DHA di dalam hasil. Kajian-kajian seterusnya telah dijalankan pada 30 ^{o}C.

Di dalam kajian berasaskan masa telah didapati bahawa nisbah DHA bertambah dengan masa kepada suatu nilai maksimum 0.750 selepas 40 minit, kemudian berkurangan secara perlahan-lahan. Apabila tindakbalas dijalankan selama 40 minit menggunakan kuantiti Lipozyme yang berbeza-beza, keputusan menunjukkan bahawa nisbah DHA yang maksimum sebanyak 0.763 diperolehi apabila 0.4 g Lipozyme digunakan bagi setiap 0.54 mmol FFA. Kuantiti Lipozyme yang lebih tinggi mengurangkan nisbah DHA di dalam hasil.

FFA-belut yang telah diperkayakan dengan DHA yang didapati selepas tindakbalas pada keadaan-keadaan optimum mengandungi 52% DHA. Ini adalah tujuh kali lebih tinggi daripada kandungan DHA di dalam FFA-belut yang asal iaitu lebih-kurang 7%.

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I certify that an Examination Committee met on 4th August 2003 to conduct the final examination of Zainal Kifli b Abdul Razak on his Master of Science thesis entitled "Enzymatic Enrichment of Docosahexaenoic Acid (DHA) in Oil from Eel (*Monopterus albus*)" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Science The members of the Supervisory Committee are as follows

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

ZAINAL KIFLI ABDUL RAZAK

Date: 28/10/2003

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LIST OF ABBREVIATIONS

AA	Arachidonic Acid
BHT	Butylated hidroxytoluene
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl ester
FFA	Free fatty acid
FID	Flame ionization detector
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
PA	Palmitic acid
PUFA	Polyunsaturated fatty acids

CHAPTER I

INTRODUCTION

During the last two decades fish oils have attracted great interest among scientist for their medicinal and nutritional properties. The benefits of fish oils are attributed to their high content of n-3 polyunsaturated fatty acids (n-3 PUFA), mainly eicosapentaenoic acid (EPA ; C20:5n-3) and docosahexaenoic acid (DHA ; C22:6n-3). These fatty acids were proven to have healing and preventive effects against coronary heart disease, cancer, asthma and arthritis (Stansby, 1990). DHA is used as supplement in infant foods as it is essential for the growth of infants (Gill and Valivety, 1997).

The PUFA composition in fish oils is affected by several factors, such as geographical location, temperature and water salinity (Greene, 1990). The oils extracted from the Northern Hemisphere coldwater fishes are rich in n-3 PUFA, especially EPA and DHA. However, the content of these fatty acids in some tropical fishes are quite comparable to those of coldwater fishes. A study on several tropical seawater fishes done by Gibson (1983) showed that a number of them contain high level of n-3 PUFA especially DHA. It is generally accepted that fishes living in low temperature waters generally contain high ratio of n-3 PUFA to n-6 PUFA as compared to tropical fishes. It is also accepted that an increase in water salinity will increase the ratio of n-3 PUFA to n-6 PUFA. With these general assumptions it is expected that

tropical freshwater fishes have low n-3 PUFA and high n-6 PUFA content. However, this might vary from species to species.

At present, EPA and DHA are produced from coldwater fishes such as menhaden, tuna, sardine and salmon. Little has been done to study the PUFA content of Malaysian freshwater fish. Endinkeau and Tan Kim Kiew (1993) studied the lipid and fatty acid content of several Malaysian freshwater fishes. The study was based on the oil extracted from the fillet of the fishes. They discovered that most of these fishes contain low level of EPA and DHA. However, the eel, *Monopterus albus* - a species commonly found in rice fields - contain a significantly high level of DHA.

Coldwater fish oils may contain up to 30% total DHA and EPA. Thus, they are widely used as food supplements. However, for pharmaceutical purpose, this level is considered low. Hence, there is a need to prepare products with a higher concentration of these fatty acids. These products would be in the form of free fatty acids (FFA), ethyl esters or tryglycerides, depending on their specific uses.

The enrichment of PUFA in fish oils could be done via a number of methods. Among them are urea complexation method (Haagsma *et al*, 1982), supercritical fluid chromatography (Alkio *et al*, 2000) and enzymatic method (Shimada *et al*, 1997). The reaction temperature is an important factor in the selection of a suitable method for PUFA enrichment. This is due

to the nature of PUFAs which are relatively unstable due to the presence of multiple bonds which are easily oxidized and polymerized especially at high temperatures. In view of this, enzymatic method is getting more popular since enzymatic reaction could be carried out at mild temperatures. In addition, enzymes are more selective and non-toxic. Moreover, mild temperature also means that the process is more environmental friendly since this can avoid the disposal of high temperature wastes.

Lipases are used to catalyze reactions involving oils and fats due to their reactivity on the ester bonds. Lipase acts by catalyzing esterification, transesterification or hydrolysis (Akoh, 1995). Some of the lipases are highly selective, which means that they are very active towards certain fatty acids or alcohols as compared to others. Normally the long chain polyunsaturated fatty acids are less preferred by these lipases. With this advantage these lipases are being used in the preparation of products with high PUFA content.

The enzymatic enrichment of PUFAs could be done by selective hydrolysis or esterification of the oils or fatty acids. However it was proven that the best method is the selective esterification of free fatty acids with a long chain alcohol. Shimada *et al* (1997) carried out DHA enrichment in FFA prepared from tuna oil through enzymatic esterification of the FFA with lauryl alcohol. The *Rhizopus delemar* lipase used showed low activity towards DHA and EPA, whereas most of other fatty acids were converted to esters.

However, the activity of this enzyme was higher towards EPA than DHA. After some time most of the EPA was also converted into esters. The residual fatty acid fraction was thus a DHA-rich FFA.

In this study, fish lipid was extracted from the body and head of *Monopterus albus* and the fatty acid composition was determined by gas chromatography after the lipid has been converted into methyl esters. FFA was then prepared from the lipid through saponification. The FFA was selectively esterified to lauryl ester by using Lipozyme, an immobilized lipase. The objective of this research is to determine the fatty acid profile of the oil extracted separately from the body and head of *Monopterus albus* and subsequently to enrich DHA in the FFA obtained from the lipid.

CHAPTER II

LITERATURE REVIEW

Lipids and Fatty Acids

Lipids are various organic compounds originated from organisms that are insoluble in water, but soluble in organic solvents. Simple lipids are often referred to as oils. Lipids are mainly composed of triacylglycerols (or tryglycerides), phospholipids, sterols and wax esters. A large amount of fatty acids are available in lipids as residues in tryglycerides, phospholipids and esters (Sikorski *et al*, 1990)

There are a few general rules that outline the structure of most natural fatty acids. Most of natural fatty acids have straight-chain structures with an even number of carbon atoms in their molecules. This common structure is related to the biosynthetic pathways by which the acids are produced in nature. The double bonds in unsaturated fatty acids are usually of *cis* configuration and are located at certain preferred positions. Most of the polyunsaturated fatty acids (PUFA) have the methylene-interrupted arrangement of double-bonds, that is, the double bonds are separated from each other by a methylene (CH_2) group (Gunstone, 1996). The structure of some natural fatty acids are shown in Scheme 1.

Linoleic Acid

Linolenic Acid

Arachidonic Acid

EPA

DHA

Scheme 1: The structure of some common natural fatty acids